

Full Length Research Paper

***In vitro* evaluation of the effects of *Polygonum orientale* L. on proliferation and differentiation of osteoblastic MC3T3-E1 cell**

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In this study, the stimulative effects of ethanol extract on proliferation and differentiation of osteoblastic MC3T3-E1 cell were examined by *in vitro* assays, including cell proliferative CCK-8 assay, cell cycle analysis assay and alkaline phosphatase (ALP) activity assay. Cell proliferation results showed that the ethanol extract from *Polygonum orientale* L. stimulated cell proliferation significantly at 0.1 to 100 µg/ml and the proportion S-phase of cells increased from 20.97 to 25.05% in osteoblastic MC3T3-E1 cells. At the same time, ethanol extracts increased alkaline phosphatase (ALP) activity of MC3T3-E1 cells (significant at 1 to 100 µg/ml). These results indicated that the herb *Polygonum orientale* L. could directly stimulate cell proliferation and differentiation of osteoblasts. These results preliminarily explored the pharmacological mechanism of *Polygonum orientale* L. to promote the curing of bone rheumatism and various fractures.

Key words: *Polygonum orientale* L., proliferation, differentiation, osteoblastic MC3T3-E1 cell, alkaline phosphatase.

INTRODUCTION

Polygonum orientale L. [(*P. orientale*), (Polygonaceae)] is a famous herbal drug in Traditional Chinese Medicines (TCMs). It is validated to treat many diseases, such as various fractures, muscle injuries, knocks and falls, rheumatism and pain from tissue swelling (Wang et al., 2006). Although it has been acclaimed that *P. orientale* is an effective herb to cure fractures in folk medicine, there are only a few preliminary studies on this herb in the literature (Lou et al., 2004). The effects of *P. orientale* on antibiosis (Zheng, 1998) and diuresis (Liu, 2001) were also researched, and positive results were obtained. Qin found *P. orientale* could improve immune of mice (Xie et al., 2005). Wang et al. (2008) studied the activity of

P. orientale anti-tumor, and found this plant was effective on leukemia, human breast cancer. However, there are no reports published on its precise mechanism of curing rheumatism and fractures based on cell or animal models until now.

The MC3T3-E1 pre-osteoblastic cell line is a widely adopted model for the research of osteogenesis and fractions *in vitro* (Quarles et al., 1999). Growth and differentiation factors contained in the culture medium stimulate these cells to undergo an increase of cell number and then differentiation of cell. During the proliferative period, these cells undergo DNA synthesis, cell division and then a logarithmically increasing phase until cells converge. At this point, the proliferation of cells halts and the differentiation of cells starts, and the content of alkaline phosphatase (ALP) increases markedly, which indicates the occurrence of mature osteoblasts (Suh et al., 2007). Several medications have

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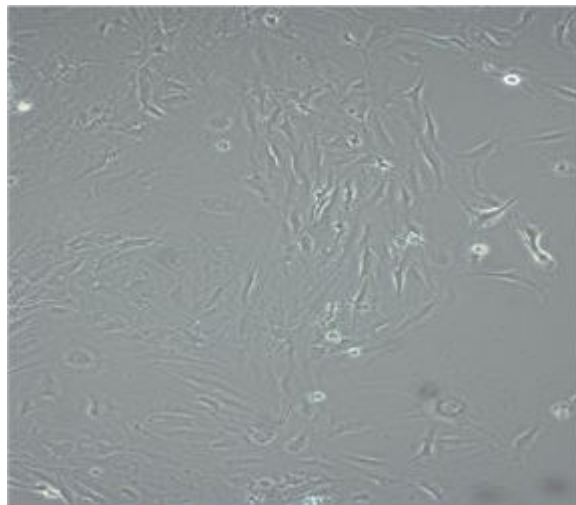


Figure 1. Cells before adding extract.

been reported to be effective for bone metabolism based on the results obtained using MC3T3-E1 cell models from compounds and extract of plant herbs, such as Icariin (Zhao et al., 2010), *Fructus psoraleae* (Song et al., 2009), *Actaea racemosa* (Chan et al., 2008), safflower seeds (Kim et al., 2008), *Ulmus davidiana planch* (Kang et al., 2006), *Drynaria fortunei* (Jeong et al., 2004), *Drynariae Rhizoma* (Jeong et al., 2005) and Soybean isoflavones (Suh et al., 2003). Recently, plants used in folk medicine have been accepted as one of the main sources of drug discovery and development. Because natural products of plant origin are still a major part of traditional medicines in western countries as an alternative source of drugs often for intractable diseases such as rheumatoid arthritis (Phillipson and Anderson, 1989). In China, traditional Chinese medicines (TCMs) represent an enormous reservoir of plants of tremendous diversity. According to the reports of literatures, great progress has been made on several medicinal herbs for treatment of fractures based on MC3T3-E1 cell line model (Han et al., 2007).

The purpose of this study is to explore the mechanism of *P. orientale* to promote the curing of bone fracture by analysing the proliferation and differentiation of osteoblastic MC3T3-E1 cell induced by its ethanol extract *in vitro*.

MATERIALS AND METHODS

Materials

MC3T3-E1 cell line was offered by Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). All other reagents came from China National Pharmaceutical Industry Corporation. Ltd. (Shanghai, China). Tissue culture media and reagents, fetal bovine serum (FBS) were from Hyclone Inc. (Logan City, Utah, USA).

Diethylstilbestrol (as positive control here) (Hu et al., 2008) was from Shanghai Sine Kangjie Pharmaceuticals Co. Ltd. (Shanghai, China).

Plant material extraction and sample preparation

The fresh root tubers of *P. orientale* were collected in Enshi Country of Hubei Province. It was authenticated by Dr. Wan Dingrong, pharmacognosy professor of School of Pharmacy, South Central University for Nationalities. The sample of *P. orientale* is also consistent with the standardized herbal specimen (No. 30068) in Institute of Drug Control of Hubei Province. The voucher specimen (SCUN08005) was deposited in the Herbarium of School of Pharmacy, SCUN. The dried root tubers of *P. orientale* (5 kg) were extracted with 95% ethanol for three times at room temperature. The combined solution was filtered and concentrated under reduced pressure to afford the 95% ethanol extract (1.2 kg). 10 mg of ethanol extract and diethylstilbestrol (as positive control) were weighed respectively, 0.1 to 100 µg/ml solutions of extract and diethylstilbestrol were got by the grade dilution.

Cell culture

MC3T3-E1 cell line was a derivative of newborn mouse calvaria, and was grown in culture flask containing DMEM supplemented with 10% FBS (Hyclone, INC., Logan City, Utah, USA, grown medium), 100 IU/ml penicillin and 100 mU/ml streptomycin in a mixture of 95% air and 5% CO₂ in a humidified incubator at 37°C (Suh et al., 2007; Han et al., 2007; Choi, 2005). These cells were planted at a density of 1×10⁵ cells/ml, the medium was refreshed daily. Experimental cultures were established in 96-well plates and cell culture flasks in presence or absence of various concentrations of sample (including ethanol extract and positive control, negative control) solutions (Kanazawa et al., 2008) (Figures 1 and 2).

Cell proliferative CCK-8 assay

The cells were seeded on 96-well plates at a concentration of 4×10³ cells/well and cultured in DMEM containing 10% FBS for 24 h to

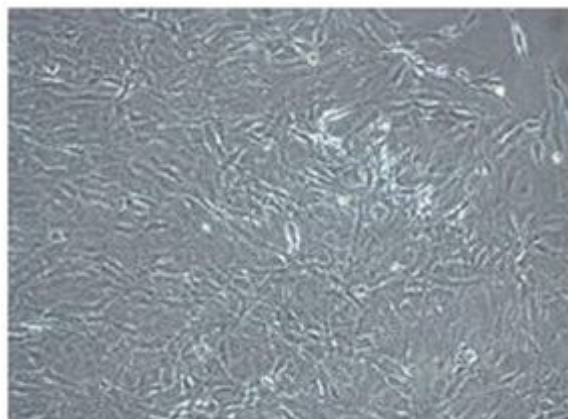


Figure 2. Cells after being treated two days by ethanol extract.

obtain adherent monolayer cell. (Peterson et al., 1996; Quarles et al., 1992; Li et al., 1995). After discarded the medium, the adherent monolayer cells were washed twice with phosphate-buffered saline (PBS), then maintained in the fresh DMEM containing various concentrations of ethanol extract solutions (Tulipano et al., 2010) for 2 days. DMEM was negative control. Wells added diethylstilbestrol solutions were set as positive controls. The cells were cultured for 7 days, then cell proliferation was evaluated by cell count kit-8 (CCK-8, Beyotime Inst Biotech., China) according to manufacturer's instructions. Briefly, 10 μ l CCK-8 was added to each well (100 μ l culture medium). After incubation for 3 h at 37°C, the treated wells were detected at 450 nm by an enzyme-linked immunosorbent assay (ELISA) plate reader (He et al., 2010). Experiments were carried out in five replicates and expressed as mean \pm S.D.

Cell cycle analysis assay

The cells (2×10^5 cells/well) were suspended in medium and seeded in 6-well plates, containing DMEM supplemented with 10% FBS, cultured for 24 h to obtain adherent monolayer cell. The fresh serum-free culture replaced the medium to synchronize them. Cells were washed with PBS, The fresh medium containing various concentrations of ethanol extract solutions was added. After 3 days, cell cycle was evaluated with cell cycle according to manufacturer's instructions, 70% cold ethanol was added slowly, and kept at 4°C until staining. The fixed cells were washed with PBS, then centrifuged 3 min 2000 rpm, then were incubated with 100 μ l RNase (1 mg/ml) for 30 min at 37°C, and then 50 μ l (1 mg/ml) propidium iodide (PI, Jingmei, Shenzhen, China) was added to cell suspension and continued to incubate at 4°C for 30 min in the dark. The cell proliferative cycle was detected by a FACSCalibur (BD Bioscience, San Jose, CA, USA) flow cytometer (Sreeja and Sreeja, 2009). The data were analyzed by Modfit software (Verity Software House, Topsham, ME, USA).

Alkaline phosphatase (ALP) activity assay

The cells were seeded in cell culture-flask at a concentration of 2×10^6 cells/ml and cultured in DMEM supplemented with 10% FBS for 7 days. The medium was replaced with phenol red-free α -MEM containing 5% charcoal-dextran-treated FBS (CD-FBS; GIBCO). Then, the cells were cultured in various concentrations of ethanol extract solutions, supplemented with 10 mM β -glycerophosphate

(β -GP) (G9422, Sigma Chemical Co., St. Louis, MO), which was added to initiate *in vitro* cell differentiation (Choi, 2005; Chae et al., 2001; Suh et al., 2007; Amos et al., 2008). After 3 days, the medium was removed and the monolayer cells were gently washed twice with PBS. The cells were digested by 0.8 ml trypsin, and then added 0.6 ml medium containing 10% FBS to stop digesting, the derivative was removed to 1.5 ml centrifugal tube, and centrifuged 20 min at the speed of 3000 rpm (Han et al., 2007). Supernatant was discarded and the PBS was added to centrifugal tube, resulting in a solution of 1×10^7 cells/ml cell concentration. Freeze-thaw cycles were repeated (-70°C refrigerator) to damage cells and to release intracellular components. Then the damaged cell solution was centrifuged for 15 min at the speed of 12000 rpm. The supernatant was used for the measurement of ALP activity (Lee et al., 2008; Isama et al., 2003) was determined using an ALP activity assay kit with an enzyme immunoassay system (R&D System Inc., Minneapolis, MN, USA) according to the manufacturer's recommendation. Taking blank wells as zero, the treated wells were detected and read absorbance at 450 nm using an enzyme-linked immunosorbent assay (ELISA) plate reader. Experiment were carried out in five replicates and expressed as mean \pm S.D.

Statistics

All experiment results were performed at least three replicates. Results were expressed as the mean plus or minus the standard deviation (S.D). Differences between groups were examined for statistical significance by the soft of GraphPad InStat. P values equal or less than 0.05 were considered statistically significant.

RESULTS

Effect of extract from *P. orientale* on proliferation of MC3T3-E1 cells

The effects extract on the proliferation of MC3T3-E1 cells were shown in Figure 3. The dose-response curves indicated that extract (1 to 100 μ g/ml) had a significant stimulation of the proliferation of MC3T3-E1 cells (* $p < 0.05$). Consequently, we could come to a conclusion that the herb could stimulate proliferation of MC3T3-E1 cells.

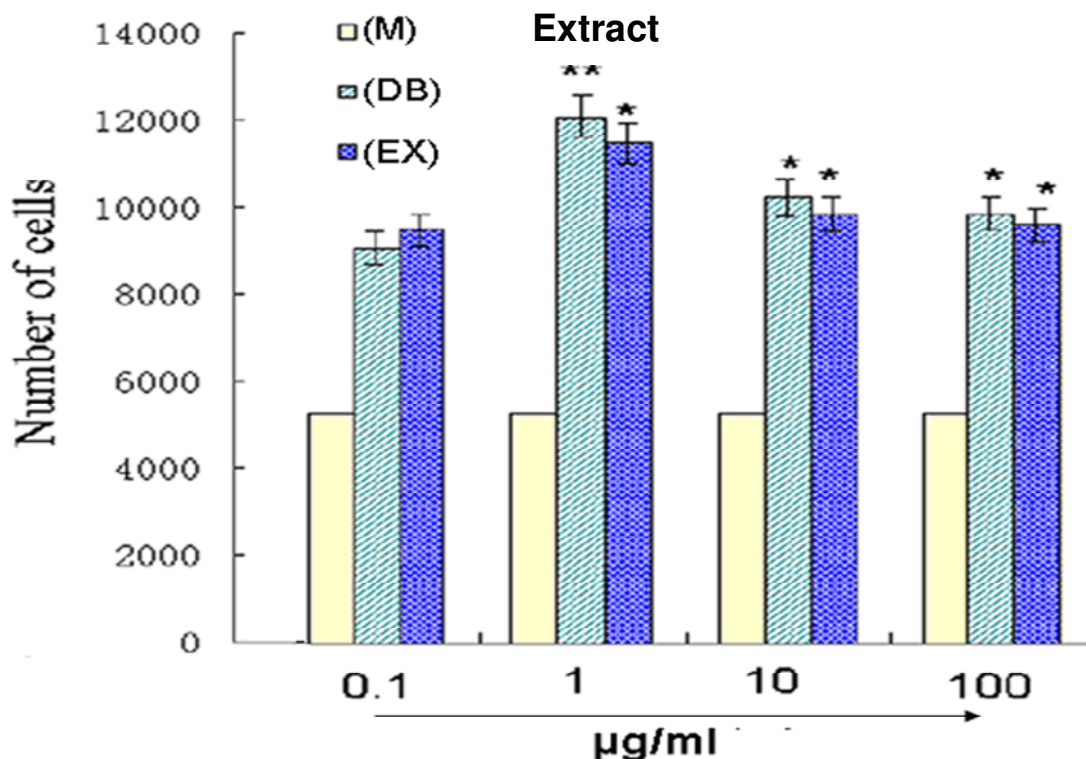


Figure 3. The extract dose-response increased the proliferation of MC3T3-E1 Cells. Cells in 96-well plates were cultured with medium and various concentrations extract (0.1 to 100 µg/ml) for three days. Each point represents the mean \pm S.D. of five determinations. * $P < 0.05$, compared with control (Medium). "DB" means positive control diethylstilbestrol; "Ex" means extract; "M" means medium.

Cell cycle analysis

Cell proliferation is regulated by cell cycle progression. After MC3T3-E1 cells were treated with various concentrations of ethanol extract, dose-dependently up-regulated the cell proportion in S-phase significantly (** $P < 0.01$). When cells were cultured with 0.1 to 100 µg/ml ethanol extract, the proportion S-phase of cells increased from 16.33 to 25.05% (Figure 4), therefore, the results indicated that ethanol extract promoted cell proliferation by up-regulating cell cycle progression.

Effects of extract of *P. orientale* on the ALP of MC3T3-E1 cells

The cure of fracture is related to the differentiation of osteoblasts. In this study, the effect of *P. orientale* on the expression of ALP, a marker for osteoblast differentiation, was detected in MC3T3-E1 cell. The effects of extract of *P. orientale* on ALP activity of MC3T3-E1 cells were shown in Figure 5. The dose-response curves indicated that ethanol extract (1 to 100 µg/ml) all had a significant positive effect on the differentiation of MC3T3-E1 cells (* $p < 0.05$, ** $p < 0.01$); Therefore, the herb has a markedly

positive effect on the differentiation of MC3T3-E1 cells.

DISCUSSION

TCMs have been developed over 5000 years and attracted increasingly attentions owing to its low toxicity and enormous natural resources. Currently, many kinds of TCMs have been investigated in detail for their more therapeutic effects. However, there were few reports on the confirmatory therapy effect and precise mechanism of these agents in cells or animals, which would be vital for exploring its curative effect.

P. orientale is well known in TCMs theory as a representative herb for its functions in clearing away heat and toxic material, dehumidification, unimpeded channels and collaterals, promote tissue regeneration and promoting granulation, and astringent. Furthermore, it has been widely used to cure various fractures, rheumatism and bones injuries (Zhang, 2008) although the precise mechanism of these agents is very poorly understood.

To investigate the effect of *P. orientale* on fractures and rheumatism, we selected the well-recognized osteoblastic MC3T3-E1 cell line as *in vitro* model (Quarlers et al.,

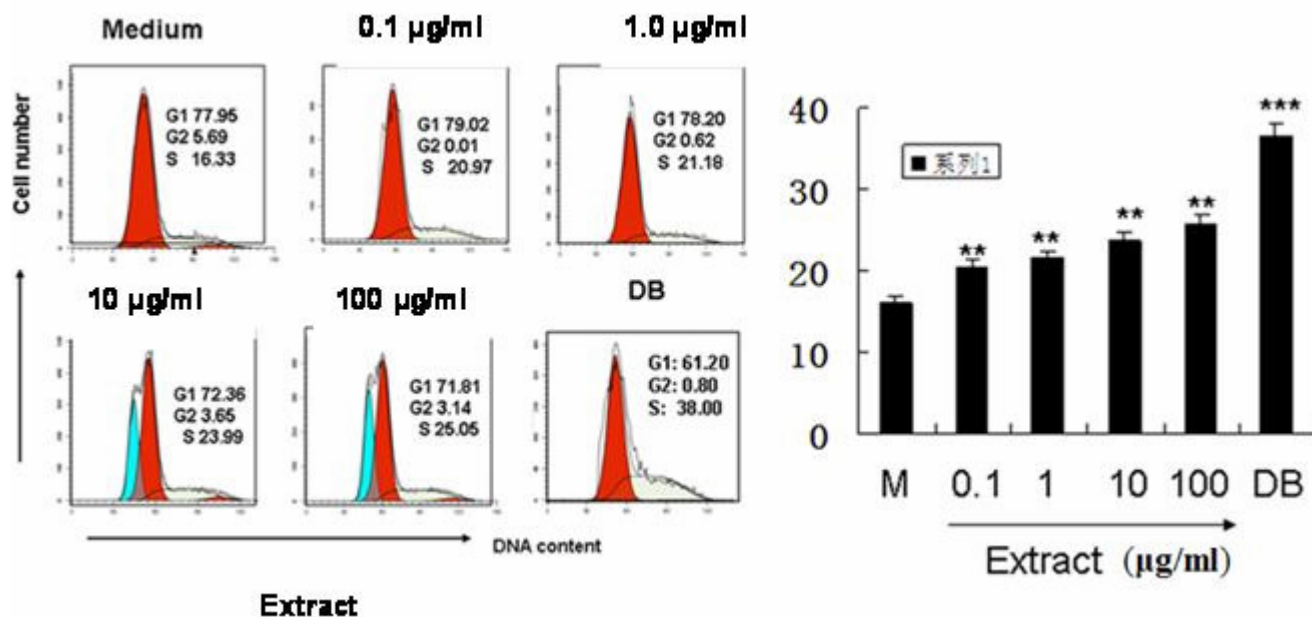


Figure 4. The results of Cell cycle analysis. MC3T3-E1 cells were seeded in six-well plates and were cultured with medium, ethanol extract (from 0.1 to 100 µg/ml) and DB (1 µg/ml) for three days. Cell cycle was analyzed by the cellular DNA flow cytometry. Results were expressed as means ± S.D. of five determinations. **P < 0.01 compared with control (Medium). "DB" means positive control diethylstilbestrol; "Ex" means extract; "M" means medium.

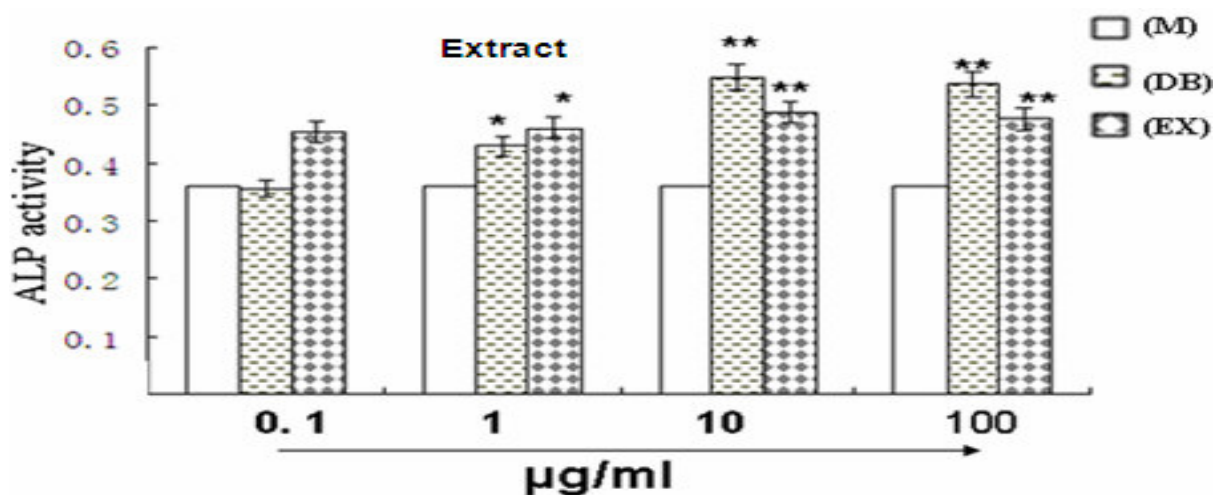


Figure 5. Effects of extract on ALP activities of MC3T3-E1 cells. MC3T3-E1 cells were cultured by medium, ethanol extract (0.1 to 100 µg/ml) and DB (0.1 to 100 µg/ml) for three days in presence 10 mM β-glycerophosphate (β-GP). Results were expressed as means ± S.D. of five determinations. *P < 0.05, **P < 0.01 compared with control (Medium) by student t-test. DB" means positive control diethylstilbestrol; "Ex" means extract; "M" means medium.

1999; Suh et al., 2007). In this study, we examined cell growth in osteoblastic MC3T3-E1 cells *in vitro*. The results showed that the low dosage of ethanol extract (1-100 µg/ml) could still stimulate cell growth. At the same time, the results of cell cycle assay demonstrated that ethanol extract could effectively increase DNA synthesis of cells in the S-phase (20.97 to 25.05%; 0.1 to 100 µg/ml), which coincides with the results of proliferation.

ALP is the most widely recognized biochemical marker for osteoblastic activity (Kim et al., 2001; Hu et al., 2008).

Although, its precise mechanism of action is poorly understood, this enzyme is believed to play an important role in bone metabolism (Yamaguchi and Gao, 1998; Shimada et al., 2009). Therefore, we examined the effects of *P. orientale* on the ALP activity of osteoblastic MC3T3-E1 cells. The results showed that ethanol extract

from *P. orientale* even at lower concentrations (1 to 100 µg/ml) could exhibit significant positive effects on the osteoblastic cell growth and ALP activity. The effects demonstrate that the root tubers of *P. orientale* could increase both ALP activity and DNA synthesis by stimulating the proliferation and differentiation of osteoblasts *in vitro*, respectively.

Therefore, these results partly elucidated the pharmacological mechanism of *P. orientale* to promote the healing of bone fractures and rheumatism.

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